ROLE OF HISTONE METHYLTRANSFERASE, "EZH2" IN HUMAN CANCER

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CERTIFICATE

This is to certify that the thesis entitled **"Role of Histone methyltransferase, "EZH2" in Human Cancer"** which is being submitted by **Mr. Rashmi Ranjan Sahu,** Roll No. **409LS2055,** for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that the thesis **entitled "Role of Histone methyltransferase, "EZH2" in Human Cancer"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafied and original research work carried out by me under the guidance and supervision of **Dr. Samir Kumar Patra,** Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:

Place: **RASHMI RANJAN SAHU**

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ABSTRACT

Epigenetic alternations significantly contribute many disease progressions as the genetic changes do. However, there is a crucial difference between genetic and epigenetic alterations, which has important implications for development of cancer treatments. Therefore it is tough duty and also necessary to identify the all pathways or mechanism by which they work for the development of strategies to impede their abnormal behaviours.

Chromatin condensation or relaxation is closely linked with tumorigenesis or cancer by regulating the pattern of gene expression. Methylation is often associated with silencing of many genes. Polycomb group proteins are important for the somatic maintenance of imprinting and for maintaining the silenced state of homeotic genes. The frequent deregulation of PcG activities in human tumors has direct oncogenic effects and results essential for cancer cell proliferation. Ezh2, a Polycomb group protein is the catalytic subunit of PRC2 having conserved SET domain targets H3K27me3. There is little contribution of Ezh2 is known till this date, still H3K27me3 and DNA methylation play a role together in gene silencing by loss of tumor suppression. Ezh2 also linked to histone deacetylation and it is shown that HDAC which is associated to EED is required for PRC2 to perform its function.

Here we investigate the functional role of EZH2 in lymph node and gall bladder cancer progression. EZH2 and SUZ12 transcripts were consistently elevated in lymph node carcinoma as compared with normal blood cell. Band intensity analysis demonstrated that EZH2 and SUZ12 protein levels were strongly associated with lymph node cancer aggressiveness. HDAC overexpression in tis cancer supports the EZH2-mediated cell invasion required an intact SET domain and histone deacetylase activity. This study provides the functional link between H3K27me3, DNMTs and HDACs in cancer aggression.

ABBREVIATIONS

E(Z):	**Enhancer of Zeste**
EZH2:	**Enhancer of Zeste homolog 2**
EED:	**Embryonic ectoderm development**
ESC:	**Extra sex combs**
HMT:	**Histone methyltransferase**
PcG:	**Polycomb group**
PRC:	**Polycomb repressive complex**
SAM:	**S-Adenosyl methionine**
SUZ:	**Suppressor of Zeste**
TrxG:	**Trithorax group**
Xi:	**X chromosome inactivation**
CpG:	**Cytosine phosphate Guanine**
DNMT:	**DNA methyltransferase**
HCAC:	**Histone deacetylase**
PCR:	**Polymerase chain reaction**
SET:	**Su(var) Enhancer of Zeste Trithorax**
HAT:	**Histone acetyltransferase**
NuRD:	**Nucleosome remodelling**
CDK:	**Cyclin dependent kinase**

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1. INTRODUCTION

1.1. Hallmarks of Cancer

Cancer has been discovered centuries ago but it remains a mystery till today. Cancer is ultimately the result of cells that uncontrollably grow and do not die. Normal cells in the body follow an orderly path of growth, division, and Programmed cell death is called apoptosis, and when this process breaks down, cancer begins to form. Unlike regular cells, cancer cells do not experience programmatic death and instead continue to grow and divide. Cancer harms the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumors (except in the case of leukemia where cancer prohibits normal blood function by abnormal cell division in the blood stream).

Hanahan and Weinberg proposed in their classic review (2000), six essential hallmarks that collectively dictate malignant growth: (1) self-sufficiency in growth signals, (2) insensitivity to growth-inhibitory signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis [1]. Recently, the same authors added two new hallmarks and two enabling characteristics, which have gained increasing importance during the progress of cancer research. The two enabling characteristics are genomic instability and tumour-promoting inflammation, while the two new hallmarks are deregulation of cellular energetics and avoidance of immune destruction.

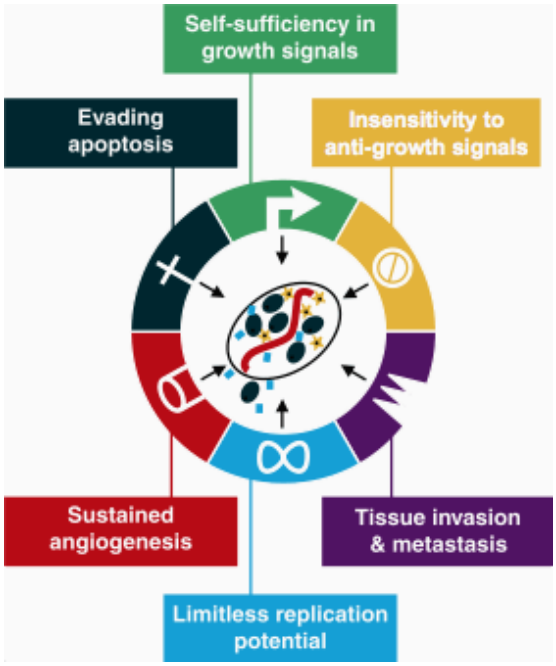


Fig: 1. Acquired capabilities of Cancer (Hanahan and Weinberg, 2000)

Epigenetic alternations significantly contribute many disease progressions as the genetic changes do. However, there is a crucial difference between genetic and epigenetic alterations, which has important implications for development of cancer treatments. Mutation results in the permanent change in the DNA sequence which is very tough to recover or counteract but the epigenetic changes is transient and can be reversed by the antagonists. Chromatin condensation or relaxation is closely linked with tumorigenesis or cancer by regulating the pattern of gene expression. Therefore it is tough duty and also necessary to identify the all pathways or mechanism by which they work for the development of strategies to impede their abnormal behaviours. The site of epigenetic alternations mainly focused on DNA methylation at CpG island but methylation of histones are also implicated in oncogenesis.

1.2. Cancer as an Epigenetic disease

Much effort has been invested in identifying genetic mutations in cancer. In inherited cancer syndromes this approach has proved successful. Furthermore, mutations early in the genesis of common cancers have also been identified and these are likely to be associated with tumour initiation. In contrast, few specific genetic mutations have been linked to tumour progression, leading Feinberg to suggest that epigenetic changes may be involved. Epigenetic changes occur without a change in the DNA sequence and they can be induced by various factors. Thus it is possible, for example, that a DNA mutation leads to cellular transformation, but induced changes in the epigenome of the transformed cell enhances the probability that it will be capable of metastasising [2].

Cancer is an epigenetic disease at the same level that it can be considered a genetic disease. In fact, epigenetic changes, particularly DNA methylation, are susceptible to change and are excellent candidates to explain how certain environmental factors may increase the risk of cancer. The delicate organization of methylation and chromatin states that regulates the normal cellular homeostasis of gene expression patterns becomes unrecognizable in the cancer cell. The genome of the transformed cell undergoes simultaneously a global genomic hypomethylation and a dense hypermethylation of the CpG islands associated with gene regulatory regions. These dramatic changes may lead to chromosomal instability, activation of endogenous parasitic sequences, loss of imprinting, illegitimate expression, aneuploidy, and mutations, and may contribute to the transcriptional silencing of tumour suppressor genes. The hypermethylation-associated inactivation affects virtually all of the pathways in the cellular network, such as DNA repair, the cell cycle, and apoptosis. The aberrant CpG island methylation can also be used as a biomarker of malignant cells and as a predictor of their behaviour, and may constitute a good target for future therapies.

In this scenario, a genetic mutation initiates the cancer but epigenetic change promotes its progression. Epigenetic processes may also be involved in cancer initiation. It is possible that epigenetic change may lead directly to cancer initiation. Alternatively, changes already induced within the epigenome may 'prime' cells in such a way as to promote cellular transformation upon a subsequent DNA mutagenic event. In this case the epigenetic component of the cancer initiation is intricately entwined with the genetic component. The involvement of epigenetic change in cancer initiation is of course not mutually exclusive to it having also a role in cancer progression.

A genetic alteration in the gene encoding an 'epigenetic enzyme' (e.g. a histone acetyltransferase, histone methyltransferase) may lead to changes within the epigenome. If, for example, these changes cause the activation of an oncogene then cancer may arise. In addition, mutations in genes that code for proteins that recognize and bind to epigenetic marks (e.g. methyl binding domain proteins and bromo/chromo domain proteins which bind to methylated DNA and acetylated/methylated histones respectively) could be as important in cancer as mutations in the enzymes themselves. Although these are genetic events that lead to cancer, an alteration in the epigenome most likely also plays a part. However, it should be noted that many of the histone-modifying enzymes also modify non-histone proteins, thus making a direct link between enzyme deregulation, changes in the epigenome and cancer extremely difficult.

DNA methylation was the first epigenetic alteration to be observed in cancer cells5. Hypermethylation of CpG islands at tumour suppressor genes switches off these genes, whereas global hypomethylation leads to genome instability and inappropriate activation of oncogenes and transposable elements1. It appears that genomic DNA methylation levels, which are maintained by DNMT enzymes, are delicately balanced within cells; over-expression of DNMTs is linked to cancer in humans, and their deletion from animals is lethal [3]. Furthermore, methyl cytosine is capable of spontaneously mutating in vivo by deamination to give thymine. Indeed, 37% of somatic p53 gene mutations (and 58% of germ-line mutations) occur at methyl CpGs and these mutations are strongly implicated in the cause of cancer [4].

The histone N-terminal histone tails are crucial in helping to maintain chromatin stability and they are subject to numerous modifications. Most modifications have some role to play in transcriptional regulation and so each has the potential to be oncogenic if deregulated deposition leads, for example, to loss of expression of a tumour suppressor gene [2,3].

Histone acetylation tends to open up chromatin structure. Accordingly, histone acetyltransferase (HATs) tend to be transcriptional activators whereas histone deacetylases (HDACs) tend to be repressors. Many HAT genes are altered in some

way in a variety of cancers [3]. For instance, the p300 HAT gene is mutated in a number of gastrointestinal tumours. On the other hand, alteration of HDAC genes in cancer seems to be far less common. However, despite this low incidence of genetic mutation in cancer, HDAC inhibitors are performing well in the clinic as anti-cancer drugs.

Histone methylation: All lysine methyltransferases that target histone N-terminal tails contain a so called SET domain. This domain possesses lysine methyltransferase activity and numerous SET domain-containing proteins are implicated in cancer [5,6]. One example is the Suv39 family of enzymes that catalyse methylation of H3K9. Transgenic mice devoid of these enzymes are very susceptible to cancer, especially B cell lymphomas. Histone demethylases have only very recently been identified and as yet no linkage to cancer has been observed. However, such a linkage seems probable.

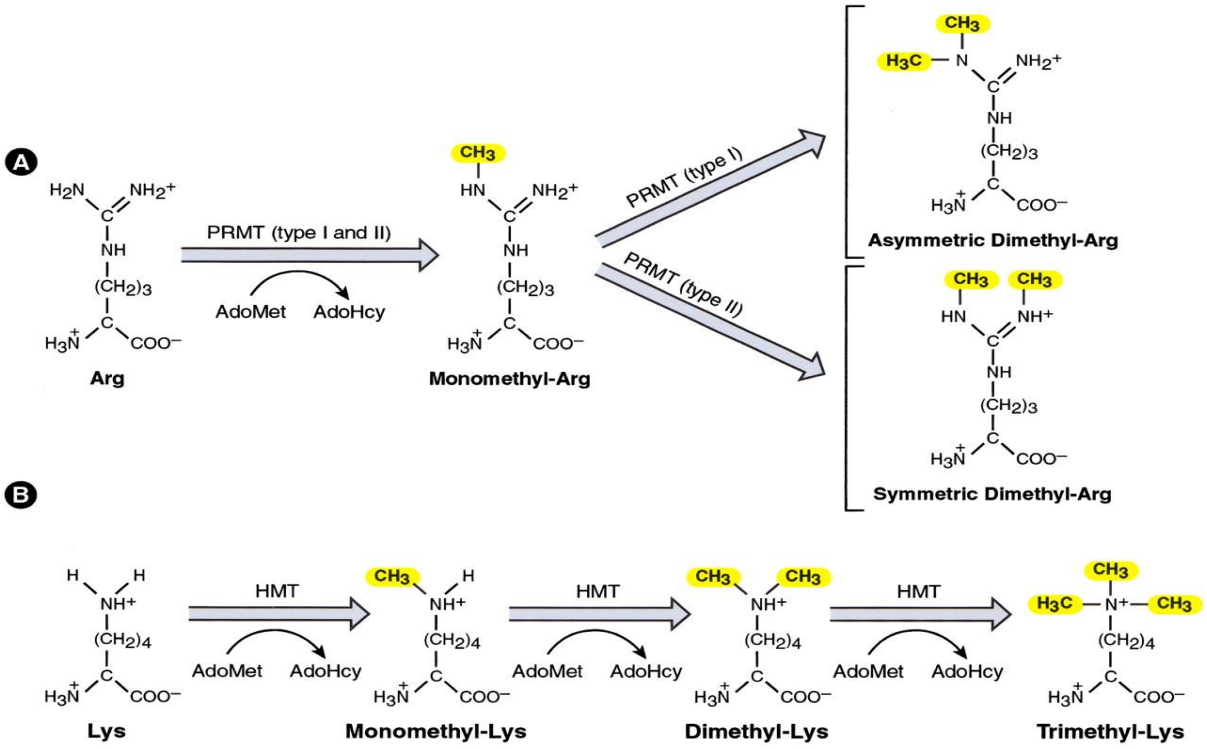


Fig: 2. Mechanism of Lysine and Arginine methylation.
http://genesdev.cshlp.org/content/15/18/2343/F2.small.gif

Histone phosphorylation: H3S10 and H3S28 are phosphorylated at mitosis - a crucial part of the cell cycle; misregulation here is often associated with cancers. Indeed, the Aurora kinases that perform this H3 phosphorylation are implicated in cancer [6].

An early event following DNA damage is the phosphorylation of H2AX, a process that is required for efficient DNA repair. If repair is not performed correctly the cell is left with damaged DNA, with predictable consequences.

1.3. Inheritance of Epigenetic marks

Epigenetic inheritance is defined as cellular information, other than the DNA sequence itself that is heritable during cell division [3]. Epigenetics affect the transcription in the cell, thereby controlling gene expression and abnormal epigenetic changes can have serious effects for the organism.

Although substantial detail about the inheritance of DNA methylation through mitotic cell division is known, any analogous mechanisms for the inheritance of histone modifications are not as clearly understood. Histone Lysine methylation does not appear to turnover quickly and presumably persists through cell division. Even for highly dynamic modifications such as acetylation, earlier studies showed that specific sites of acetylation are maintained during mitosis, and such inheritance of acetylation patterns is thought to maintain the expression profiles of genes through successive generations [7,8].

Early studies using radioactively labelled histones strongly suggested that the parental histones are transferred as intact octamers, and are randomly segregated onto the two daughter DNA strands [9,10]. Nucleosome assembly complexes then deposit additional newly synthesized histones to fill in the gaps. Interestingly, a recent report suggested that the parental nucleosomes may actually divide in a semiconservative manner whereby the parental histone octamer is split into H2A-H2B/H3-H4 heterodimers that are then equally segregated onto the two daughter DNA strands [11]. In this scenario, the nucleosome assembly complex then deposits newly synthesized histones to complete the pre-existing half of the nucleosome. This idea is intriguing because it invokes the possibility of a mechanism that can faithfully and equally transmit histone-associated information from parent to daughter DNA strands. However, it is not clear how this hypothesis fits in with earlier data that showed transfer of intact histone octamers during DNA replication.

At present, a similar process for replicating histone modification has not been shown. Nevertheless, it is interesting to note that some HMTs such as EZH2 also contain chromodomain motifs that potentially have a role in targeting these enzymes to selectively modified regions of chromatin. Also, several HATs such as CBP and p300/CBP associated factor also contain bromodomains, a motif that has been shown to have acetyl-Lys binding properties [12]. Whereas the chromo and bromodomains of these HMTs and HATs have not yet been found to bind specific methylated or

acetylated histones, the functional significance of these potential modification binding motifs present on histone methyl and acetyltransferases is yet out of reach.

1.4. Histone methylation and Chromatin structure

Methylation plays dual roles in regulating histone accessibility as it has been linked to both activation and silencing of transcription [13]. Methylation confers additional levels of complexity as methylation can be mono, di- or tri- methylation and occurs at lysine (K) and arginine residues. Methylation of arginine residues has been linked to gene activation while lysine methylation has been linked with gene silencing and activation [14,15]. Arginine is a positively charged amino acid and the nitrogen of arginine can be modified by the addition of one or two methyl groups. Currently eight mammalian protein arginine methyltransferases have been identified. While the mechanism through which arginine methylation enhances transcription remains unknown, evidence exists that methylated arginines collaborate with other transcriptional activators and enhance their activity to promote transcriptional activation. Methylation on lysine residues generally correlates with gene suppression but can also contribute to gene activation. One, two or three methyl groups can be added by histone methyltransferases (HMTases). Trimethylation of histone 3 lysines 9 and 27 or histone 4 lysine 20 35 is associated with gene silencing, while H3K4 di- and trimethylation, H3K36 trimethylation, and H3K79 di- and trimethylation methylation contributes to gene activation [16].

In higher eukaryotes, histone methylation is involved in the maintenance of cellular identity during somatic development. During spermatogenesis, most nucleosomes are replaced by protamine. Therefore, it is unclear if histone modifications function in paternal transmission of epigenetic information. Here we show that active H3K4 di-methylation (H3K4me2) and repressive H3K27 trimethylation (H3K27me3), two modifications important for Trithorax and Polycomb-mediated gene regulation, are present in chromatin of human spermatozoa and show methylation-specific distributions at regulatory regions. H3K4me2-marked promoters control gene functions in spermatogenesis and cellular homeostasis suggesting that this mark reflects germline transcription. In contrast, H3K27me3 marks promoters of key developmental regulators in sperm as in soma. Many H3K27me3-marked genes are never expressed in the male and female germline, and in early “totipotent” embryos, suggesting a function for Polycomb in repressing somatic determinants across generations. Targets of H3K4me2 and H3K27me3 are also modified in mouse spermatozoa, implicating an evolutionary conserved role for histone methylation in chromatin inheritance via the male germline.

The histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2) belongs to Polycomb Group (PcG) and is involved in gene repression [17]. EZH2 catalyzes

trimethylation of H3K9 and H3K27 with a strong preference for K27 [18]. EZH2 has a cysteine rich SET domain which is involved in binding to the Polycomb repressive complex [18]. EZH2 binds to the CIITA proximal promoter and is a "master regulator" of the silencing histone modifications at CIITApIV, thus implicating roles for EZH2 in regulating other silenced, but inducible genes. Of note are recent studies indicating expression of EZH2 is elevated in multiple human cancers, including breast cancer.

While the mechanisms by which histone methylation contributes to gene silencing are not known, cross talk between various modifications has been observed (Muller and Verrijzer 2009). H3K4 methylation blocks methylation of H3K9 and induces the dimethylation of H3K27, resulting in an opening of chromatin structure [19]. H3K36 methylation recruits histone deacetylases and re-establishes closed structure resulting in decreased gene expression. Similar to reversible acetylation, histone methylation can also be reversed by histone demethylases (HDMTs). The first identified HDMT is LSD1 which demethylates mono- and di- H3K4 in a flavin adenine dinucleotide (FAD) dependent oxidative reaction. This observation led to discovery of highly conserved Jumonji C containing proteins which remove methyl groups from lysine residues. This recent observation that methylation is reversible by histone demethylases provides new levels of regulation for gene expression. There are also additional interactions between methylated histones and the DNA methylation machinery which further determines the availability of DNA for gene expression. While histone methylation and the impact on DNA structures is reversible, DNA methylation is permanent and leads to stable repression of gene expression. The interactions between methylated histones and methylated DNA are complex as histone methylation mediates DNA methylation and DNA methylation also serves as a template for modifications to histones. Greater understanding of the crosstalk between modifications to histones and modifications to DNA provide new, and largely unexplored, levels of regulation of gene.

1.5. Role of SET domain in histone methylation

Structural-functional analysis of Suv39H1 showed that its HMT activities are mediated by the SET domain of the proteins. This highly conserved domain is found in large number of proteins from yeast to human, and is named after the three founding proteins that share this domain: Su(VAR)3–9, enhancer of Zeste [E(Z)], and trithorax (TRX) [22]. Indeed, all three of these proteins, as well as a growing number of other SET domain-containing proteins, have now been shown to have HMT activities and they each have exquisite specificity toward different sites on H3 or H4 [24]. E(Z) and TRX belong to the Polycomb (Pc)- and trx-group gene product families that have previously been identified as chromatin-modifying proteins

important for maintaining the balanced expression of homeotic genes in Drosophila and mammals. The human EZH2 methylates histone H3 at Lys27, whereas the TRX homologs [for example, Set1p in S. cerevisiae, TRX, TRR (trithorax-related) in Drosophila, and MLL (myeloid or mixed lineage leukemia) in human] all methylate H3 at Lys4 [24]. Consistent with the previously assigned roles of the Pc and trx complexes in transcriptional repression and activation, respectively, H3 Lys27 methylation has been correlated with transcription silencing, and H3 Lys4 methylation is now well established as a chromatin mark for active genes. Analogous to the binding of the chromodomain of HP1 to Lys9-methylated H3, the chromodomain of the Pc protein has been found to bind Lys27-methylated H3, and functions to recruit the Pc complex to initiate Pc-dependent transcriptional silencing [26]. Interestingly, swapping of the chromodomain of HP1 and Pc was sufficient to switch the nuclear localization of these proteins in Drosophila S2 cells, indicating that the binding of the respective chromodomain of these proteins to the Lys9- or Lys27-methylated H3 has important roles in the targeting of chromatin-binding proteins in vivo.

H3 Lys4 methylation has been well documented to be associated with euchromatic regions in diverse organisms including S. pombe, Drosophila, and mammalian cells; however, a direct role for this modification in activating transcription has not been found. Biochemical pull-down assays showed that Lys4- methylated H3 can bind to the chromatin remodelling enzyme Isw1p [26]. In addition, association of Isw1p to chromatin is dependent on the H3 Lys4-methylating enzyme Set1p in S. cerevisiae, suggesting that this modification promotes association of remodelling factors that in turn facilitate transcription. Biochemical data also suggested that Lys4-methylated H3 can prevent binding of the mammalian HDAC complex NuRD (nucleosome remodelling and HDAC) to chromatin [25]. The consensus so far suggests that H3 Lys4 methylation may have an indirect regulatory role by maintaining the associated genomic regions in a state that is poised for transcription activation.

1.6. Role of Su(var) in histone methylation

It has long been known that histones, particularly H3 and H4, are methylated at a number of lysine (Lys) and arginine (Arg) residues. The major sites of Lys-methylation on histones identified so far are: Lys4, Lys9, Lys27, Lys36, Lys79 on H3 and Lys20 on H4 [20,21]. In addition, the Lys residue can be methylated in the form of mono-, di-, or trimethylation, and this differential methylation provides further functional diversity to each site of Lys methylation. A major breakthrough in the understanding of H3 Lys-methylation function was the discovery that one of the well-studied Su(var) genes in fact encodes a histone methyltransferase (HMT). The Su(var)3–9 gene in Drosophila, and its homolog, Clr4, in fission yeast S. pombe, were

originally identified by genetics screens to have roles in transcriptional silencing associated with heterochromatin (in Drosophila) and mating type silencing (in S. pombe) [22]. Biochemical analyses of the human homolog, Suv39H1, revealed that this protein has an enzymatic activity that specifically methylates histone H3 at Lys9 [23]. This finding, followed by a convergence of genetics and biochemical data, and aided by the development of antibodies highly specific for H3 methylated at different sites, quickly delineated a pathway of heterochromatin formation.

In mouse, disruption of the two Su(var)3–9 homologs, Suv39h1 and Suv39h2, led to impaired viability and chromosomal instabilities in the double-null mouse embryos [24]. By using antibodies that specifically distinguish the mono-, di- and trimethylation state of Lys9- methylated H3, it was found that fibroblasts derived from the dn embryos show a specific loss of the Lys9 trimethylated form of H3 at pericentric heterochromatin [25]. Interestingly, whereas HP1 localization in these cells is compromised, the presence of condensed chromatin at pericentric regions, as indicated by DAPI dense staining, appears to be unaffected [25, 26].

2. REVIEW OF LITERATURE

2.1. Housekeeping functions of EZH2

Polycomb group (PcG) and trithorax group (trxG) proteins are well known to be part of the cellular memory system [27,28]. Both groups of proteins are involved in maintaining the spatial patterns of homeotic box (Hox) gene expression that are established early in embryonic development by the transient expression of segmentation genes. In general, PcG proteins are transcriptional repressors that maintain the _off state', whereas trxG proteins are transcriptional activators that maintain the _on state'. Recent demonstrations that members of PcG and trxG proteins contain intrinsic histone methyltransferase (HMTase) activity raise the possibility that PcG and trxG proteins participate in cellular memory through methylating core histones [29].

2.1.1. Hox gene silencing

The role of EZH2/EED complex, which includes histone deacetylation in transcriptional repression, is well documented. It has long been established that this complex can, for example, silencing of Hox genes. The EED-EZH2 complex, containing the core subunits EZH2, EED, SUZ12, and RbAp48, functions as a histone H3K27-specific methyltransferase. Here we describe the identification and characterization of a related EED-EZH2 protein complex which is distinguished from the previous complex by the presence of another PcG protein, hPHF1. Consistent with the ability of hPHF1 to stimulate the enzymatic activity of the core EED/EZH2 complex in vitro, manipulation of mPcl1, the mouse counterpart of hPHF1, in NIH 3T3 cells and cells of the mouse male germ cell line GC1spg results in global alteration of H3K27me2 and H3K27me3 levels and Hox gene expression. Small interfering RNA-mediated knockdown of mPcl1 affects association of the EED/EZH2 complex with certain Hox genes, such as HoxA10, as well as Hox gene expression concomitant with an alteration on the H3K27me2 levels of the corresponding promoters [30]. Therefore, our results reveal hPHF1 as a component of a novel EED/EZH2 complex and demonstrate its important role in H3K27 methylation and Hox gene silencing.

2.1.2. X-chromosome inactivation

Another most important function of EZH2 is X-chromosome inactivation, is a developmentally regulated process involving choice of the active X chromosome, initiation of silencing on the Xi and maintenance of silencing throughout all subsequent cell divisions [31]. Xist, a noncoding RNA transcript, coats the Xi and is

thought to mediate its silencing [32]. Studies over the past several years have shown that several epigenetic modifications,

including histone H4 hypoacetylation, enrichment of the variant histone macroH2A and DNA methylation, are features of the Xi [32]. An EED–EZH2 histone methyltransferase complex was first implicated in X-inactivation when mice homozygous for an EED mutation were found to be defective in maintaining X-inactivation in extraembryonic, but not embryonic, tissues [31]. Consistent with a role of the EED–Ezh2 complex in X-inactivation in cells of extraembryonic lineage, EED and Ezh2 were found to be enriched on the Xist-coated Xi in trophoblast stem cells [32], an extraembryonic cell type. Two recent studies have further evaluated the role of EED–Ezh2-mediated H3- K27 methylation in X-inactivation [33].

Biochemical and genetic studies have provided compelling evidence that Drosophila PcG proteins function in two distinct protein complexes: the Polycomb repressive complex 1 (PRC1) and the Extra sex combs and Enhancer of Zeste (ESC–E[Z]) complex (also known as PRC2), although the dynamic compositions of the complexes are still controversial [33]. Similar protein complexes have also been described in mammals [34.35], underscoring the functional conservation of the PcG proteins during evolution. The discovery that the ESC–E[Z] complex (known as the EED–EZH2 complex in mammals) contains intrinsic HMTase activity prompted the analysis of the role of histone methylation mediated by EED–EZH2 or ESC– E(Z) in PcG silencing, X-inactivation, germline and stem cell development, and cancer. Here we review recent progress in our understanding of the biochemical and biological functions of this complex and its associated enzymatic activity in these cellular processes.

2.1.3. Stem cell pluripotency

Recent studies suggest that Ezh2 and its associated H3-K27 methyltransferase activity might be important for maintaining stem cell pluripotency. Stem cells are characterized by their capacity for self-renewal and their ability to differentiate into all cell types. Characterization of stem cells has shown that several protein factors, including the POU domain homeobox transcription factor Oct3/4, are essential for stem cell pluripotency [36]. Oct3/4 is one of the earliest expressed transcription factors and is crucial for murine development at the preimplantation stage. It is expressed at high levels in stem cells such as embryonic germ cells, embryonic stem cells and embryonic carcinoma cells, but it undergoes rapid repression when these cells start to differentiate [37]. The first indication that murine Ezh2 might be linked to stem cell pluripotency came from the observation that Ezh2 is essential for the derivation of pluripotent embryonic stem cells [36]. Given the early embryonic lethal

phenotype of Ezh2 mutants, and the fact that Ezh2 is a maternally inherited protein, the function of Ezh2 in preimplantation development is difficult to address.

2.1.4. Germ line development

The EZH2/EED complex is present throughout early preimplantation development in germ cells, oocytes and pluripotent stem cells in mice. The role of this protein complex is yet unclear. Here MES-2 (counterpart of Ezh2), MES-3 and MES-6 (counterpart of EED) functions as a protein complex for transcriptional silencing. Recent studies reveal that MES-2/3/6 complex is responsible for H3K27me3, as this case with EZH2/EED complex in mice and human. Mutations in mes gene showed a marked alteration in H3K27me3 marks, which may explain the transcriptional silencing in the germ cell lineage. This PcG complex is also detected in mouse germ cell lineage from the time of specification and later when the germ cell lineage is established. The precise role of this protein in regulating the epigenetic status and maintenance of germ cell lineage remains to be elucidated [37].

2.2. EZH2 in Epigenetic scenario

PcG proteins appear to perform their functions by forming complexes. Two distinct Polycomb complexes have been characterized by immunoprecipitation, yeast two-hybrid and size-fractionation experiments in mammalian system. BMI- 1, RING1, HPH1/2/3, and HPC1/2/3 [38] proteins constitute the Polycomb Repressive Complex 1 (PRC1) and EZH2, EED, SUZ12, RbAp46/48 and AEBP2 (E(z), Esc, Su(z)12, and RbAp48 in Drosophila) [38] make the Polycomb Repressive Complex 2 (PRC2). The PRC2 was shown to physically associate with Yin Yang 1 (YY1, the human homolog of Pho in Drosophila) [39], which is the only known DNA binding protein of the Polycomb group, while all the others do not have apparent DNA binding motifs [40]. However, YY1 binding sites alone are not sufficient to alter the epigenetic pattern. It is shown that HDAC which is associated to EED [41] is required for PRC2 to perform its function. EZH2/E(z) contains a conserved histone methyltransferase domain, SET domain, named after SU(var)3-9, E(z) and Trithorax which contain this enzymatic domain [40]. Based on recent discoveries, a model is proposed to illustrate how Polycomb group complexes perform their function. At the beginning, the histone tails on the chromatin are acetylated and target genes are transcriptionally active. Once the cellular memory is disturbed, through the cell signaling pathway, PRC2 receives the signal from the cell signaling pathway and binds to a PRE. HDAC is then recruited to the PRE to deacetylate the histone tails, so that PRC2 can methylate the histone tails. This alters chromatin structure and enables PRC2 and HDAC to access the target gene promoters. Further, the histone tails on the gene promoters are deacetylated and then

methylated. This methylation establishes a binding site for the N-terminal chromodomain of PcG proteins so that PRC1 is recruited to the promoters of target genes to repress their expression by repressing transcription initiation [42,43].

Accordingly, PRC2 is also called PRCi (initiation), and PRC1 is called PRCm (maintenance). Human E(z) homolog EZH2 was initially identified as a protein associated with proto-oncogene VAV in lymphoma. The gene of EZH2 maps to chromosome 7q35 and consists of 20 exons, encoding 746 amino acid residues. By yeast two-hybrid screen with EZH2, human Esc homolog EED was identified to interact with EZH2 in vitro, and by co-IP, EZH2 and EED were confirmed to form a complex in vivo. Several studies demonstrated that the WD40 domains of EED are essential for this EZH2-EED complex, while the point mutants in the WD40 domain blocked the interaction between EZH2 and EED. Another polycomb protein SUZ12 is also characterized as an essential component of PRC2 for its HMTase enzymatic activity. Several interacting partners of the EZH2-EED complex include SUZ12, RbAp48 and AEBP2, which were identified by isolating and characterizing the enzymatic complex which had high HMTase activity to histone H3[44]. The reconstituted complex of EZH2, EED, SUZ12, RbAp48 and AEBP2 can specifically methylate Histone H3 in vitro.

In 2002, Kuzmichev et al. found that PRC2 exhibited HMTase activity and could specifically methylate H3K9 and H3K27 [36]. Furthermore they proved that the methylation of H3K27 provides a mark for PRC1 protein PC1 binding, therefore, PRC1 is recruited to the targets. Generally the PRC2 protein EZH2 preferentially methylates Lysine 27 on histone 3 (H3K27) [38,39]. But under certain conditions, it can also methylate other substrates, such as H3K9 and H1BK26. Because EZH2 is the only known histone methyltransferase which can tri-methylate H3K27, the level of tri-me-H3K27 is used as the marker of EZH2 enzymatic activity. Some groups also reported that there exist Polycomb Repressive Complex 3 (PRC3) and Polycomb Repressive Complex 4 (PRC4) in cells [43,44]. The human EED has four different isoforms due to alternate translation initiation sites from the same mRNA. All of these isoforms can associate to EZH2 to form different complexes and bind to their substrates. EED1 (the largest isoform) and EZH2 form PRC2 (~400-kDa complex) and methylate H3K27 in the presence of histone H1. EED3 and EED4 (the two shortest isoforms) can form PRC3 (~400-kDa complex) with EZH2, and methylate H3K27 when histone 1 is absent. EED2, SirT1, which specifically binds to EED2, and EZH2 form PRC4 (~1.5-MDa complex) and methylate K26 residue on H1B.

2.3. Cancer links to PRC2 subunits

Less is known about cancer-associated alterations in PRC2 subunits besides EZH2, EED over-expression in human cancers has not been widely reported and the studies

that first documented EZH2 over-expression in prostate and breast cancer revealed unchanged EED levels in the same patient samples [45]. There are also examples of SUZ12 alterations in cancer tissues including overexpression in colon, breast and liver tumors [45–47]. In addition, SUZ12 is implicated in endometrial cancer since a chromosome rearrangement creating a SUZ12 fusion protein is frequently associated with endometrial stromal tumors [46]. Finally, over-expression of PCL3, which is a homolog of the PRC2-associated protein, PHF1, is also associated with many cancers including colon, skin, lung and liver [47]. Since consequences of EZH2 overabundance in cancer cells are still emerging, it is an open question if excessive levels of these non-catalytic partners work through similar mechanisms.

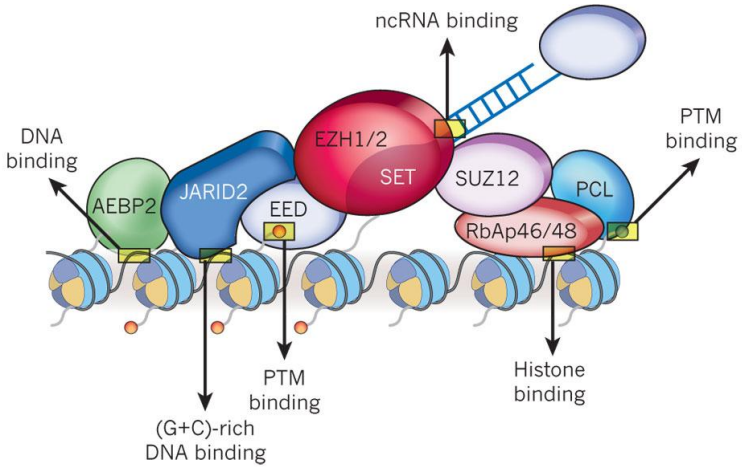


Fig: 3. Polycomb Repressive Complex 2 (PRC2) binds to the PRE and function to repress the target gene. (www.nature.com/nature/journal/v469/n7330/images/nature09784-f3.2.jpg)

2.4. EZH2 mediates DNA methylation

Epigenetics, the heritable regulation of gene expression independent of DNA sequence, is emerging as an essential aspect of the mechanisms of cell lineage determination and maintenance. DNA methylation and histone modification controls the epigenetic phenomena, particularly histone methylation. Current studies show the requirement of histone methylation for DNA methylation which is interconnected.

Recently, Vire et al. reported that EZH2 can directly control DNA methylation, so that the two processes which can repress gene expression [39], Histone methylation and DNA methylation are connected, and this finding elucidates a mechanism by which Polycomb Group and DNA methyltransferases (DNMTs) work together to repress gene expression. The authors demonstrated that the complex pulled down with GST-EZH2 possesses DNMT activity and this complex contains EZH2, DNMT1, DNMT3A and DNMT3B, and that the N-terminal H-1 and H-II domains of EZH2 are

required for establishing and maintaining this complex. Furthermore, reciprocal coimmunoprecipitation (co-IP) confirmed that DNMTs interact with PRC2, EZH2 and EED in vivo. Notably, similar to the effect of DNA methylation inhibitor 5'-azadeoxycytidine treatment, knock-down of EZH2, DNMT1, DNMT3A or DNMT3B markedly increases expression of their target genes, but not of the housekeeping genes. By ChIP in EZH2 RNAi cells, the authors demonstrated that EZH2 is essential for DNMTs recruiting to the promoters of their targets, while RNA polymerase II could interact with the promoters and turn on the expression of target genes.

Several papers reported that PcG proteins are involved in the de novo DNA methylation in cancers or cancer cell lines. By ChIP analyses, it is shown that genes with DNA methylation in cancer are marked with Polycomb proteins. Interestingly,

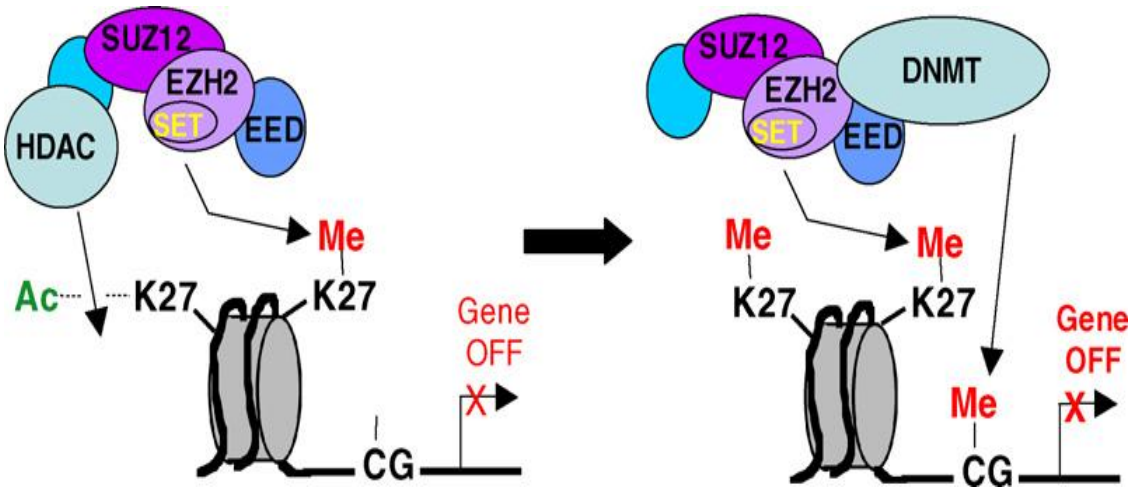


Fig: 4. Recruitment of HDAC and DNMTs by PRC2 for repression of target genes. (www.nature.com/nature/journal/v469/n7330/images/nature05344-f3.2.jpg)

many tumor suppressors are repressed by active de novo DNA methylation, and are pre-marked with H3K27 tri-methylation, indicating the mechanism by which EZH2 and PRC2 promote cancer progression. In normal tissues, some tumor suppressors are marked with H3K27 tri-methylation, but not de novo DNA methylation, demonstrating that EZH2 alone is not sufficient for DNA methylation, and several other components have to be recruited to methylate DNA to facilitate gene repression. Although EZH2 is required for DNA methylation, it is not required for maintaining DNA methylation and keeping the genes off. Some evidence demonstrated that knock-down of EZH2 can de-repress genes whose promoters are methylated, such as MYT1 and WNT1; But for the genes whose promoters are hypermethylated, such as MLH1, knockdown of EZH2 could not increase their expression, even though the H3K27 trimethylation level is decreased because of the EZH2 knockdown[47].

2.5. EZH2 and cancer

It has been reported that increased expression of EZH2 had been associated previously with invasive growth and aggressive clinical behaviour in prostate and breast cancer (Su et al., 2003). Amounts of both EZH2 mRNA and EZH2 protein were increased in metastatic prostate cancer. In addition, clinically localized prostate cancers that expressed higher concentrations of EZH2 showed a poorer prognosis. Thus, Varambally et al. concluded that dysregulated expression of EZH2 may be involved in the progression of prostate cancer as well as being a marker that distinguishes indo- lent prostate cancer from those at risk of lethal progression (Dohner et al., 1998). EZH2 mRNA transcript and protein levels were elevated in invasive and metastatic breast cancer when compared to normal breast tissues (Rhodes et al., 2003). Higher EZH2 protein levels in the breast cancer were associated with a shorter disease- free interval after initial surgical treatment, lower overall survival, and a high probability of disease-specific death. Furthermore, the high EZH2 levels were strongly associated with poor clinical out- come in breast cancer patients (Kleer et al., 2003). In 2005, Sudo et al. reported that EZH2 expression was significantly higher in human liver cancer cell lines and tissue specimens compared to normal sections (Sudo et al., 2005). Later, Chen et al. demonstrated that knock-down of EZH2 markedly inhibited the growth of hepatocellular carcinoma (HCC) cells and reduced the tumorigenicity of HCC cells in the nude xenograft mice. In bladder and gastric cancers, EZH2 was increased at both the mRNA and protein levels in cancer cell lines and cancer tissue specimens relative to normal controls. Several reports have been demonstrated that there is an inverse correlation between EZH2 and E-cadherin, which is a vital gene for controlling tumor invasion. Further studies indicated that E-cadherin is key media- tor among the list genes that downstream of EZH2 regulation (Cao et al., 2008).

2.5.1. EZH2 in Prostate cancer

Prostate cancer is a leading cause of cancer-related death in males and is second only to lung cancer. Although effective surgical and radiation treatments exist for clinically localized prostate cancer, metastatic prostate cancer remains essentially incurable.

Polycomb group protein enhancer of Zeste homolog 2 (EZH2) is over one of the earliest reports was a gene profiling study where EZH2 was scored as the most significant gene up-regulated in metastatic prostate cancer compared to clinically localized prostate cancer [35]. This study also showed that loss of EZH2 inhibits growth of prostate cancer cells. Similar requirements for EZH2 in proliferation of other cell types have been described [44]. Significantly, EZH2 over-expression in prostate cell lines led to silencing of a discrete set of >100 target genes, which was dependent upon an intact SET domain [35]. Thus, this work suggested that EZH2

overabundance alters the genomic expression program through chromatin hypersilencing. Statistical analysis also revealed that EZH2 levels could provide a valuable prognostic indicator of patient outcome [35] and subsequent studies have described the prognostic value of combined sets of prostate markers that include EZH2 overabundance [50].More recently, a Polycomb repression "signature", consisting of a cohort of 14 repressed EZH2 target genes, has been described as a tool for predicting prostate and breast cancer patient outcomes [50].

2.5.2. EZH2 in Breast cancer

The role of EZH2 in breast cancer and demonstrated that EZH2 is elevated at both the transcript and protein levels in invasive and metastatic breast cancer when compared to normal breast tissues [51]. Immunohistochemical analyses performed on a spectrum of breast cancer tissues demonstrated that high EZH2 levels were strongly associated with poor clinical outcome in patients. Higher EZH2 protein levels were associated with a shorter disease-free interval after initial surgical treatment, lower overall survival, and a high probability of disease-specific death (i.e. death due to breast cancer). Also, high EZH2 expression was associated with disease-specific death in patients with lymph node-negative disease, but not in patients with positive lymph nodes. EZH2 expression was associated with disease-specific survival in patients with stage I and II disease, but not in patients with advanced stage (stages III and IV). Kaplan–Meier analysis showed that EZH2 levels were strongly associated with bad outcome in both ER positive and -negative invasive carcinomas suggesting that EZH2 has prognostic utility independent of ER status.

Importantly, overexpression of EZH2 could increase HDAC enzymatic activity. Also overexpression of EZH2, but not the dominant negative mutant of EZH2, EZH2ΔSET, promotes anchorage-independent growth in epithelial cells [51]. The expression patterns of PRC1 and PRC2 are different in various stages of tumor progression. The PRC1 complex expression is always high in normal breast tissues, pervasive lesions and invasive breast carcinomas, which are consistent with the function of PRC1 to maintain the gene expression pattern. But in normal breast tissues, EZH2 and EED are rarely detectable except in the cycling cells.

2.6. Applications of EZH2

Synthetic peptide fragments of EZH2 were able to stimulate peripheral blood mononuclear cells (PBMCs) and produce EZH2-specific cytotoxic T lymphocytes (CTLs). Interestingly, those EZH2-specific CTLs can generate IgG against EZH2 and are toxic to HLA-A24 positive cells [46]. This finding provides an alternative approach to inhibit EZH2 function in cancer progression.

Cyclin-dependent kinase 1/2(CDK1/2)-mediated Thr 350 phosphorylation as an important mechanism in control of EZH2-mediated epigenetic gene silencing in cancer cells. In turn, blockage of Thr 350 phosphorylation diminishes the global effect of EZH2 on gene silencing (Chen et al.) This discovery motivated us to find or design the inhibitors that can dephosphorylation of EZH2 to apply in the fields of cancer therapy. In context of Chen et al. report, CDK1/2 inhibitors could be served as a selective option for cancer therapy that blockage of phosphorylation of EZH2 [47].

Knockdown of EZH2 suppresses development of Ewing tumors in association with up regulation of genes involved in neuroectodermal/endothelial differentiation. These findings seem to indicate a key role for EZH2 in maintaining an undifferentiated phenotype in Ewing tumor. On the other hand, EZH2 is crucial in regulating cell cycle via the retinoblastoma pathway. 11,12,67,68 Knockdown of EZH2 deregulates genes involved in G2/M transition in a way that inhibits cell proliferation by inducing G2/M arrest [47]. The findings indicate that EZH2 overexpression results in decreased BRCA1 with high levels of Cdc2- CyclinB1 complex, which drives mitosis and uncontrolled proliferation.

2.7. Targeting EZH2 for cancer therapy

The enzymatic activity and its function as an epigenetic repressor along with its established role in cancer progression, makes EZH2 an attractive target for cancer therapy. S. Varambally et.al, shown that knock-down of EZH2 inhibits cancer cell growth, motility, invasion and tumorigenesis. Small interfering RNA against EZH2 reduced EZH2 expression in several cancer cell lines, significantly inhibited cell proliferation and the cells were arrested at the G2/M phase [35]. In addition, the EZH2 knock down cells did not retain their invasive potential when injected into mice. This was apparent from the lack in tumor formation [52]. Interestingly, tumor size was decreased and growth inhibited in established tumors in mice when a shRNA against EZH2 was delivered. This indicates the promise that EZH2 holds as a candidate for cancer therapy [52].

Small interfering RNA (siRNA) duplexes4 targeted against EZH2 reduce the amounts of EZH2 protein present in prostate cells and also inhibit cell proliferation in vitro. Ectopic expression of EZH2 in prostate cells induces transcriptional repression of a specific cohort of genes. Small interfering RNA (siRNA) specific for EZH2 with the sequence GAGGUUCAGACGAGCUGAU, which has been previously identified by us to efficiently knockdown endogenous EZH2 expression in human cancer cells.

miR-101 could interact with 3’UTR of EZH2, we examined if the EZH2 transcript and protein levels can be repressed by miR101 overexpression. miR101 down regulates EZH2 protein expression in prostate cell line as well as breast cell line which have high endogenous EZH2 expression [35].

3. OBJECTIVE

As mentioned above that the level of expression of EZH2 is often high in many cancers like prostate, breast, liver, colon etc. Deregulation of PcG activities in human tumors has direct oncogenic effects and results essential for cancer cell proliferation. The mechanistic contribution of the PRC2 complex is not clear till this day; still H3K27me3 and DNA methylation play a role together in gene silencing by loss of tumor suppression.

To investigate the role of EZH2 in H3K27me3 which leads to the recruitment of HDACs and DNMTs for the hypermethylation of the promoter of the target genes we have to measure the expression level of the particular gene of the enzyme.

So my objective is **"to compare the expression level of Histone methyltransferase-EZH2 and its partner SUZ12 in cancer tissues and normal tissue".**

4. MATERIALS AND METHODS

The Human blood was collected from CWS Hospital, Rourkela as normal human tissue and Gall bladder and Lymph node cancer tissues were collected from Calcutta Medical College, Kolkata.

4.1. Total RNA isolation

Reagents and Buffers:-

- TRIzol Reagents (Sigma),
- Choloroform,
- Isopropanol,
- Ethanol (70%),
- Denaturation Buffer
  - ✓ 50 % deionized formamide,
  - ✓ 2.2 M formaldehyde,
  - ✓ MOPS buffer (pH 7.0),
  - ✓ 6.6 % glycerol,
  - ✓ 0.5 % bromphenol,
- Ethidium Bromide (EtBr),
- Agarose

Protocol:-

- ✓ 50-100 mg of tissue in a 2 ml tube with 1 ml TRIzol was transferred.
- ✓ Homogenized for 60 sec in the polytron.
- ✓ 200 µl chloroform was added.
- ✓ It was mixed by inverting the tube for 15 sec.
- ✓ Incubated for 3 min at room temperature.
- ✓ Centrifuged at 12.000 g for 15 min.
- ✓ The aqueous phase was transferred into a fresh Eppendorf tube.
- ✓ 500 µl isopropanol was added.
- ✓ Centrifuged at max. 12.000 g for 10 min in the cold room.
- ✓ The pellet was washed with 500 µl 70 % ethanol.
- ✓ Centrifuged at max. 7.500 g for 5 min in the cold room.
- ✓ The pellet was dried on air for 10 min.
- ✓ Then the pellet was dissolved in 50-100 µl DEPC-H_2O.
- ✓ Incubated for 10 min at 60° C.
- ✓ Spectrophotometric reading was taken.
- ✓ Analysed the RNA on a MOPS gel:
  - ➢ 1-3 µg RNA was dissolved in 11 µl denaturation buffer.

- 1 µl Ethidium bromide (1mg/ml) was added and denatured at 65° C for 15 min
- 1 % agarose gel was loaded in MOPS buffer plus 5 % formaldehyde.
- The gel was run at 40 V for 4 h.

4.2. cDNA synthesis (rt-PCR)

Regents and Buffer:-

- 5X First Strand Buffer
- 10mM dNTP Set
- 0.1M DTT
- Random Primers
- RNase OUT Ribonuclease Inhibitor
- Super Script II RNase H- Reverse Transcriptase

Protocol:-

- ✓ 8µl of total RNA were taken.
- ✓ Then 3 µl Random Primers was added.
- ✓ 1 µl dNTP mix was added.
- ✓ Then vortex and spin downed tube.
- ✓ Incubated at 65°C for 5 min.
- ✓ Placed tube on ice.
- ✓ 4 µl 5X Buffer, 2 µl DTT and 1µl RNAseOut were added.
- ✓ Then vortex and spin downed tube.
- ✓ Incubated at 42°C for 1 min.
- ✓ 1µl SuperScript II RNase H- Reverse Transcriptase was added.
- ✓ Incubated at 42°C for 60 min.
- ✓ Incubated at 70°C for 15 min.
- ✓ 180 µl of molecular grade water was added.
- ✓ Nanodrop 1000 was used to measure concentration. Set sample typesetting to Other Sample and the constant to 33.
- ✓ Stored at -80°C.

4.3. Gene specific PCR

Used Primers:-

Gene	Forward	Reverse
EZH2	5'-GCAGCCTTGTGACAGTTCGT -3'	5'-CAGATGGTGCCAGCAATAGA -3'
SUZ12	5′-ATTGCCCTTGGTGTACTCTGA-3′	5′-GACATGCTTGCTTTTGTTCGT-3′
β- actin	5′-CCTGTACGCCAACACAGTGC-3′	5′-ATACTCCTGCTTGCTGATCC-3′

(Tang H. *et al.*, 2009)

PCR mixture:- (Total 25µl)

- ✓ 0.2 µM dNTP- 0.5µl
- ✓ 1.5 mM $MgCl_2$- 1.5µl
- ✓ 1x PCR Buffer- 2.5µl
- ✓ Taq Polymearse (5U/µl)
- ✓ Primer 0.2 µM- 0.5µl
- ✓ cDNA- 2µl
- ✓ Mili-Q water- 17µl

PCR Conditions:-

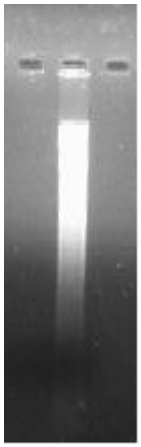
Event	Temperature (^oC)	Time
Denaturation	94	1 min
2nd Denaturation	94	20 sec
Annealing	57	20 sec
Extension	72	30 sec
Final Extension	72	5 min

5. RESULTS

Concentration and Purity of total extracted RNA:-

Tissue	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
Blood	423.90	1.21	0.78
Gall bladder	561.34	1.43	0.66
Lymph node	511.09	1.18	0.92

Agarose gel analysis of RNA:



[Total RNA in 1% agarose gel]

[Total RNA in denanuration gel]

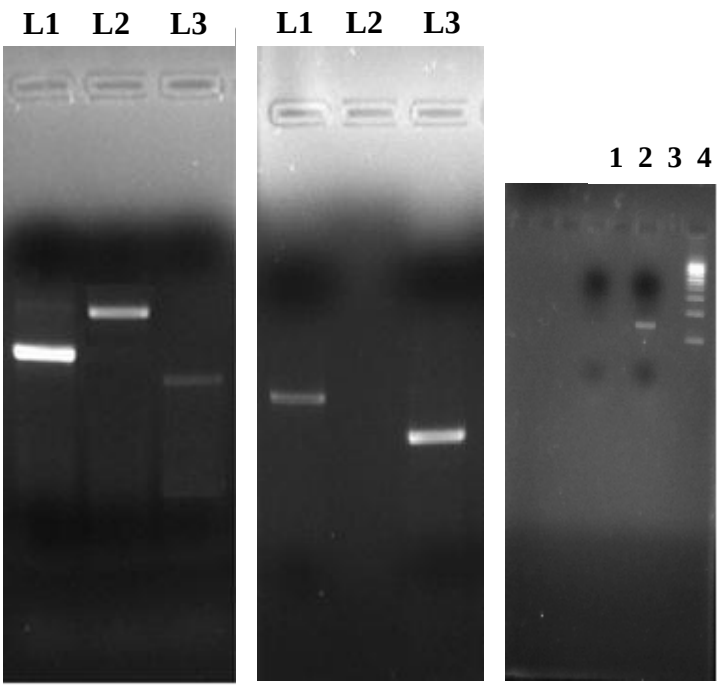
Concentration of cDNA after PCR:-

Genes	Conc^{n}. (µg/ml)		
	Blood	Gall bladder	Lymph node
EZH2	193.25	267.63	344.52
SUZ12	206.12	259.02	325.79
β- actin	221.34	218.09	213.88

Purity of cDNA after PCR:-

Geness	Blood	Gall bladder	Lymph node
EZH2	0.80	1.03	1.34
SUZ12	0.78	0.96	1.22
β- actin	0.99	0.89	0.76

Agarose gel analysis of cDNA after PCR:

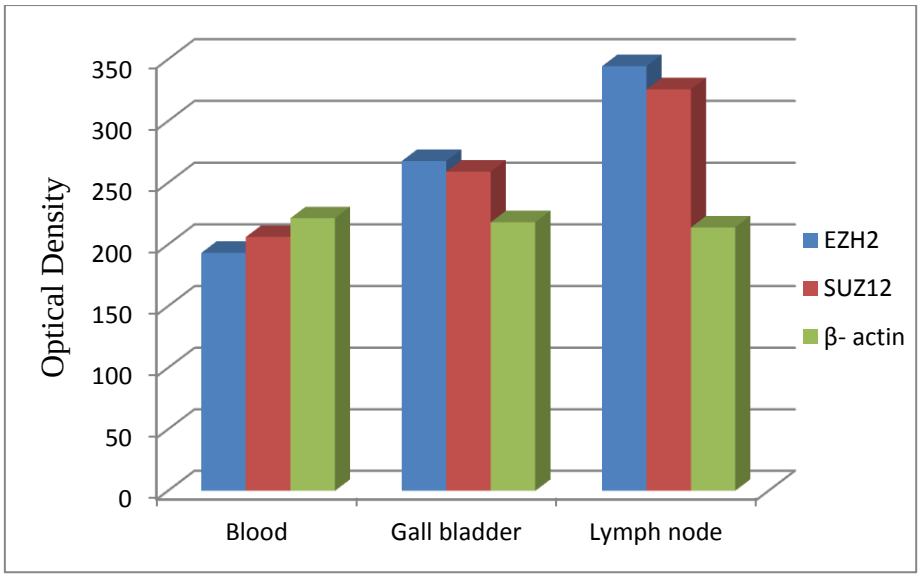


A. L1- β- actin
L2- EZH2
L3- SUZ12
B. L1- EZH2
L2- SUZ12
L3- β- actin
C. L1- EZH2
L2- β- actin
L3- SUZ12
L4- DNA ladder

A
Lymph node

B
Gall bladder

C
Blood



Graph showing expression level of EZH2, SUZ12 and β- actin in normal and cancer cells.

6. DISCUSSIONS

EZH2 and SUZ12 expressions are elevated in Lymph node Cancer:-

It was well established fact that EZH2 over expressed in case of prostate and breast cancer, so we were interested in determining whether PcG complex (EZH2 and SUZ12) is dysregulated in lymph node cancer. Peripheral blood was taken as normal tissue and the expression level was compared with lymph node tumors, and we found that EZH2 and SUZ12 transcript was overexpressed significantly in lymph node tumor cells relative to normal.

In agarose the band intensity of EZH2 in lymph node tumor is much higher as compare to blood and the spectrophotometry reveals that the expression level of catalytic subunit of PcG (EZH2) and its partner SUZ12 were overexpressed as their concentrations were 344.52 and 325.79 respectively.

From the above result we can hypothesize that due to overexpression of these PcG complexes might be playing role in invasive lymph node cancer.

Expressions of PcG complexes are normal in Gall bladder Cancer:-

The band intensities of PcG complexes in case of gall bladder cancer were not so bright as compared to normal blood cells. The concentration of PcG transcripts were 267.63 and 259.02 which was just above the normal expression range. This data reveals that PcG complexes are not overexpressed in case of gall bladder cancer.

From the above result we can say that there is no abnormal role of PcG complexes EZH2 and SUZ12 in case of gall bladder and the aggressiveness might be due to other factors.

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